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SHORT COMMUNICATION

Cell mediated and humoral immune responses of white-tailed deer experimentally infected with *Mycobacterium bovis*

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SUMMARY

The objective of this study was to improve the understanding of immune responses of whitetailed deer (*Odocoileus virginianus*) infected with *Mycobacterium bovis*. Ten mature, female, white-tailed deer were inoculated by intratonsilar instillation of 2×10^3 or 2×10^5 colony-forming units of *M. bovis*. Lymphocyte proliferation and humoral response to *M. bovis* PPD and the *M. bovis* protein, MPB70 were measured. Deer were tested for exposure to *M. bovis* by the comparative cervical skin test. Biopsy specimens of skin test sites were examined microscopically and immunohistochemically. The comparative cervical skin test correctly identified all *M. bovis*-inoculated deer as exposed to *M. bovis*. Lymphocyte proliferative responses to MPB70 were more consistent than responses to *M. bovis* PPD in *M. bovis*-inoculated deer. Antibody responses were more prominent in deer with disseminated disease than in deer with localised disease. The cellular components of delayed-type hypersensitivity reactions at skin test sites were similar to tuberculin reactions in other species. T lymphocytes of the γ/δ phenotype were seen in increased numbers in *M. bovis* PPD injection sites.

CERVIDAE are susceptible to infection with a variety of mycobacterial agents including *Mycobacterium bovis*, the causative agent of bovine tuberculosis. Recent growth of the farmed deer industry as well as an outbreak of tuberculosis in free-ranging white-tailed deer (*Odocoileus virginianus*) (Schmitt et al 1997) underscore the need for improved antemortem diagnosis of tuberculosis in deer.

Protective immunity to tuberculosis is primarily cell-mediated (North 1974). Diagnostic tests for tuberculosis in Cervidae measure cell-mediated responses, humoral responses, or both. In the US, approved tests currently include intradermal testing by the single cervical test (SCT) or the comparative cervical test (CCT) using purified protein derivative (PPD) of *M. bovis* or *M. bovis* and *M. avium*, respectively. Sensitivity and specificity for the CCT range from 80 to 91·4 per cent, and 61·3 to 98·7 per cent, respectively (Corrin et al 1993, Stuart et al 1988).

Also approved in the US for diagnosis of tuberculosis in Cervidae is the blood tuberculosis test (BTB). The BTB combines results of a lymphocyte proliferation assay with that of an ELISA (Buchan et al 1992). Antigens include PPD of *M. bovis* and *M. avium* as well as the *M. bovis* specific protein MPB70 (Harboe and Nagai 1984, Fifis et al 1989). The BTB has a sensitivity and specificity of 95·9 and 98 per cent, respectively (Griffin et al 1994).

Information on tuberculosis in white-tailed deer is limited and such reports have not addressed the host immune response to infection with *M. bovis*. The current study was designed to evaluate humoral and cell-mediated responses in a population of white-tailed deer in which infection status and duration of infection were known.

Mature, female deer were obtained from a herd with no history of *M. bovis* infection. *M. bovis* strain 1315, isolated from a white-tailed deer in Michigan, was used for inoculation. The isolate was prepared for inoculation as described (Palmer et al 1999).

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Deer were randomly assigned to one of three groups. The low dose group (n = 4), and high dose group (n = 4), received 2×10^3 and 2×10^5 colony-forming units (CFU) of *M. bovis*, respectively, instilled in the tonsilar crypts. Control deer (n = 2) received sterile saline. Deer were housed separately according to dosage group.

Eighty-seven days after inoculation, deer were humanely euthanatized. Specimens for bacteriologic culture and microscopic analysis were collected as described (Palmer 1999). Lesion distribution was characterised as localised (lesions in tonsils, oropharyngeal lymph nodes, thoracic lymph nodes, or lung), or disseminated (lesions in sites additional to those seen in localised disease).

A peptide corresponding to amino acids 26–43 of MPB70 (Radford et al 1990) was synthesised and coupled to Keyhole Lympet Hemocyanin for use in our assay by a commercial supplier (Multiple Peptide Systems, San Diego, CA). Lymphocyte proliferation assays were done on blood mononuclear cells (BMC) collected prior to inoculation and at 7, 21, 35, 49, 63 and 77 days after inoculation. Blood mononuclear cells were isolated, stimulated, and analysed as described (Stevens et al 1996) using 100 μl of various concentrations (10 to 0·01 μg) of *M. bovis* PPD (Commonwealth Serum Laboratories, Victoria, Australia), or the MPB70 peptide. Proliferation results were expressed as mean ± SEM counts per minute (CPM).

For the kinetic ELISA (KELA) assay, blood was collected prior to inoculation and on days 7, 14, 21, 35, 49, 63, 80, and 87 after inoculation. Antibodies to *M. bovis* PPD and MPB70 were measured as described (Barlough et al 1983). Antigen consisted of 100 μl of *M. bovis* PPD $(0{\cdot}5\,\mu g\ ml)^{-1}$ or recombinant MPB70 $(0{\cdot}25\,\mu g\ ml)^{-1}$ (Central Veterinary Laboratory, Weybridge, UK). Negative control samples consisted of pooled sera from TB-free deer. Positive control samples were obtained from white-tailed deer with confirmed tuberculosis.

Nine days prior to inoculation, and 81 days after inoculation, all deer were tested for exposure to *M. bovis* by the comparative cervical test (CCT) as described in the USDA guidelines, and results used to classify deer as negative, suspect, or reactor (United States Department of Agriculture 1997). A 6-mm skin punch biopsy of

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the test site was obtained with half placed in neutral buffered 10 per cent formalin and half embedded in optimal cutting temperature compound and frozen in ethanol and dry ice. Formalin-fixed sections were stained with H&E for microscopic evaluation and with a Giemsa stain for the identification of mast cell granules. Immunohistochemical staining of 5-μm cryostat sections was conducted using a commercially available kit (Histomark, Kirkegaard & Perry Laboratories, Gaithersburg, Md.). Antibody to bovine γ/δ T-lymphocyte surface antigen was produced and used as described (Kunkle et al 1995). Antibodies to bovine γ/δ T cells have previously been shown to identify γ/δ T-cells from red deer (*Cervus elaphus*) (Buchan et al 1992).

For statistical analysis, CPM from lymphocyte proliferation assays were evaluated as the logarithm of the CPM value. Differences in proliferation for each sample were compared to preinoculation proliferation levels by use of a paired t-test, or to proliferation values measured in control deer by a student's t-test. The mean KELA slope for each group at each time point was compared to a 95 per cent confidence interval generated by evaluation of responses of all deer at three sampling times prior to inoculation. Differences were considered significant at $P < 0{\cdot}05$.

No gross or microscopic lesions were seen in control deer or in one deer in the low dose group that died of aspiration pneumonia 14 days after inoculation. Bacteriologic culture and postmortem exam results are reported elsewhere (Palmer et al 1999). One of three deer in the low dose group and two of four deer in the high dose group had localised disease, while two of three deer in the low dose group and two of four deer in the high dose group had disseminated disease.

The low dose group had significantly greater proliferation than the high dose or control groups to *M. bovis* PPD at 77 days after inoculation. The low dose group also had significantly greater proliferation than the control group to MPB70 at days 7, 49 and 63 after inoculation. Proliferation to MPB70 in the high dose group was significantly greater than controls at days 21, 49 and 63 after inoculation (Fig 1). However, due to clotting of blood samples the response at 63 days after inoculation in the high dose group is based on one deer only. No differences were seen in proliferation to *M. bovis* PPD or MPB70 between deer with disseminated disease and deer with localised disease.

Antibody responses between high and low dose groups to *M. bovis* PPD or MPB70 were not significantly different. However, deer in the high dose group had significantly greater antibody responses than control deer to *M. bovis* PPD, at 21, 49, 63, 80, and 87 days after inoculation. Deer in the low dose group had greater antibody responses than control deer to *M. bovis* PPD at 80 and 87 days after inoculation (Fig 1).

Fourteen to 87 days after inoculation, antibody responses were higher in deer with disseminated disease than deer with localized disease to *M. bovis* PPD and MPB70, however, this difference was not significant.

Changes in skin thickness were greater at *M. bovis* PPD injected sites than at *M. avium* PPD injected sites in all *M. bovis*-inoculated deer (Table 1). Interpretation of skin thickness changes classified all *M. bovis*-inoculated deer as reactors. One control deer was also classified as a reactor.

Microscopic evaluation of biopsies from skin test sites of *M. bovis*-inoculated deer revealed severe inflammation in sites injected with *M. bovis* PPD. Such sites contained moderate to severe perivascular infiltrates of lymphocytes extending through the dermis, subcutis, and panniculus layers. Low numbers of perivascular lymphocytes were labeled by the bovine γ/δ antibody. Sites injected with *M. avium* PPD were normal or had mild superficial dermal perivascular infiltrates of lymphocytes. Biopsies from one control deer were normal while those of the control deer with increased change in skin thickness had mild to moderate superficial infiltrates of lymphocytes at both *M. bovis* PPD and *M. avium* PPD injection sites. Giemsa staining for mast cell granules revealed rare mast cells in *M. bovis* PPD or *M. avium* PPD injected sites.

Comparative cervical testing identified all *M. bovis*-inoculated deer 81 days after inoculation. However, the CCT also identified one control deer as *M. bovis* exposed. *Mycobacterium gastri*, a mycobacteria not belonging to the *M. tuberculosis* complex was isolated from this deer (Palmer et al 1999). False positive skin test results due to infection with saprophytic or nonpathogenic strains of mycobacteria are more common in Cervidae than in cattle (Kollias et al 1982, de Lisle and Havill 1985, Griffin 1988, Buchan and Griffin 1990, Quigley et al 1997).

In the present study, proliferative responses in both dosage groups were more consistent to MPB70 than to *M. bovis* PPD. Previously, accuracy of the lymphocyte proliferation assay has been improved by using MPB70 as the antigen (Griffin et al 1991). This protein is a major antigen produced by many strains of *M. bovis*, including strains isolated from cattle (Harboe and Nagai 1984, Fifis et al 1989).

Antibody responses in our deer did not correlate with inoculum dosage. This may be due to the short course of the study, low numbers of animals in each treatment group, or ambiguous differences between groups due to infection of one control animal with *M. gastri*. However, antibody response to *M. bovis* PPD or MPB70 was most prominent in deer with disseminated disease. Other studies in deer (Griffin et al 1991) and humans (Lenzini et al 1977, Bhatnagar et al 1977) have found antibody assays most effective in identification of individuals with caseous or liquefactive lesions in multiple organs (Griffin et al 1991). High levels of circulating antibody are thought to correlate with failure of the immune system to contain progression of disease (Lenzini et al 1977).

Descriptions of tuberculin skin test sites in experimentally infected cattle include vascular changes such as thrombosis of large veins (Feldman and Fitch 1937). Thromboses and increased numbers of mast cells are described in reactions to tuberculin in sensitized humans (Dvorak et al 1974). Thromboses were not seen in our deer, and special staining of biopsy sections did not reveal increased numbers of mast cells. Differences between the morphologic changes in deer, cattle, and humans may be due to species differences in immune response, skin test site, tuberculin, or tuberculin dose.

In ruminants, high numbers of circulating T lymphocytes may be of the γ/δ phenotype (Hein and Mackay 1991), and in other species γ/δ T cells are known to colonize epithelial surfaces including the epidermis (Born et al 1994). In mice, γ/δ T cells accu-

TABLE 1: Change in skin thickness (rounded to the nearest 0·5 mm) at comparative cervical test sites of white-tailed deer 81 days after intratonsilar inoculation of either 2×10^3 CFU (low dose) or 2×10^5 CFU (high dose) of M. bovis or saline (control).

	Deer number								
	Low dose group			High dose group				Control group	
Antigen	7	8	10	1	2	5	6	4	9
M. avium PPD	2·5	8·0	5·0	4·5	9·0	4·0	3·0	6·5	14·0
M. bovis PPD	6·0	16·0	16·0	13·0	20·0	12·0	13·0	4·0	16·0

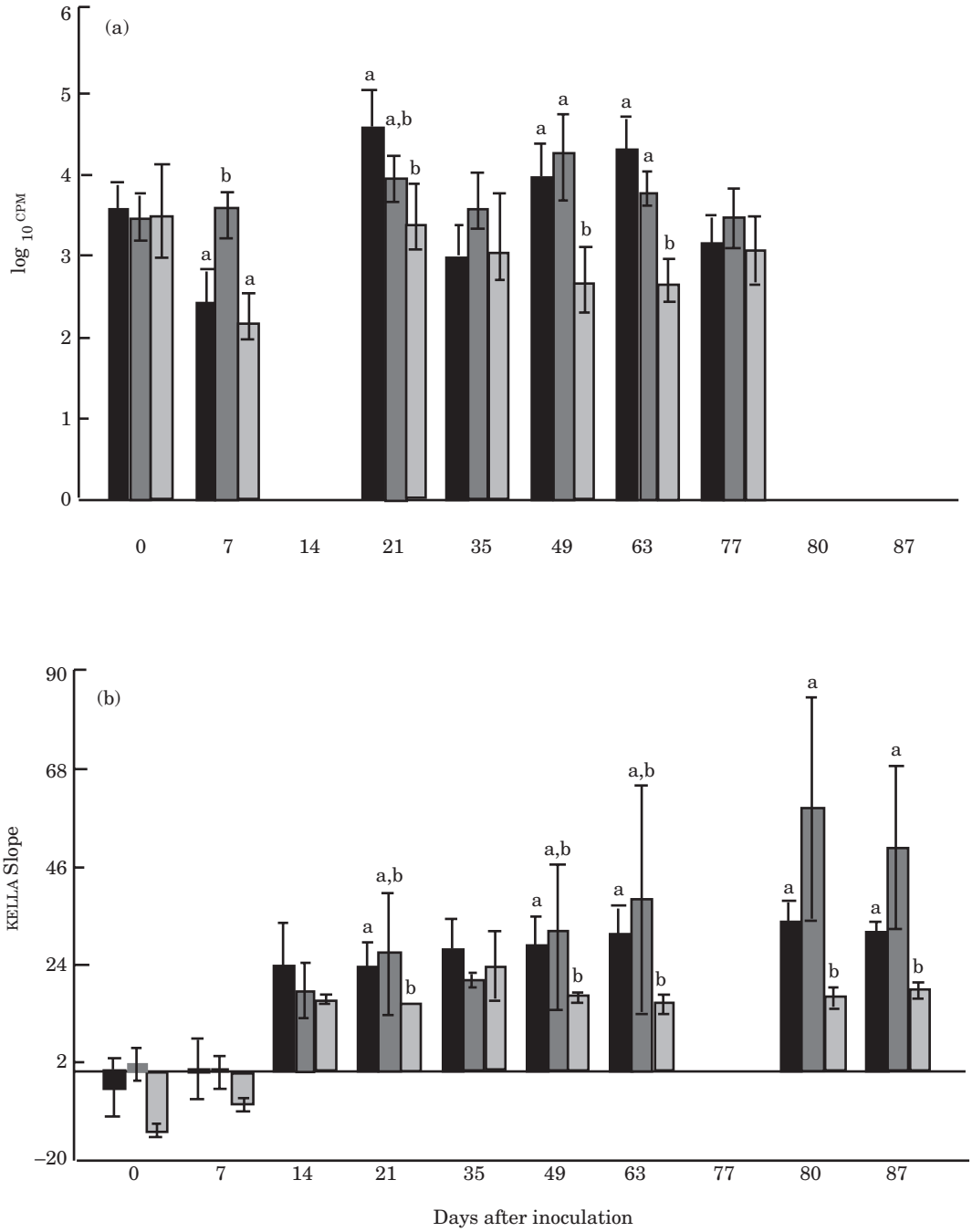

FIG 1: Lymphocyte proliferative responses to MPB70 (a) or antibody responses to *M bovis* PPD (b) of white-tailed deer after intratonsilar inoculation with 2 × 10^3 CFU of *M bovis* (low dose), 2 × 10^5 CFU of *M. bovis* (high dose) or saline (control). Lymphocyte proliferation data are presented as mean $\log_{10}$ counts/minute ± SEM. KELA results are presented as mean slope ± SEM. Means with different superscripts differ significantly ($P < 0{\cdot}05$). Key: ■, high dose; ■ low dose; ■, control.

mulate at the site of injection of *M. tuberculosis* (Janis et al 1989), and *M. bovis* BCG (Inoue et al 1991), as well as in the lung after aerosolisation of an *M. tuberculosis* extract (Augustin et al 1989). In the present study, γ/δ T cells were not seen within the epidermis; however, they were present in increased numbers in perivascular locations within *M. bovis* PPD injection sites in *M. bovis* -inoculated deer. It is clear that γ/δ T cells are recruited to the site of DTH reactions in *M. bovis* -inoculated deer, however, their role in such reactions remains unclear.

The results of this study will aid in the understanding of the immune response of white-tailed deer to *M. bovis* and assist in the antemortem diagnosis of tuberculosis in this species.

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Names are necessary to report factually on available data, however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

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